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Research Article

Molecular Genotyping of *Bacillus anthracis* Strains from Georgia and Northeastern Part of Turkey

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Abstract

Bacillus anthracis is the causal agent of anthrax and has a history of use as a biological weapon. Anthrax cases occur worldwide and the disease is endemic in certain regions. Here we describe a study of the genetic diversity of *B. anthracis* strains in two endemic areas: The country of Georgia and the Kars region of Turkey. Thirty Turkish isolates and thirty Georgian isolates were subjected to Single Nucleotide Polymorphism (SNP) sub typing, followed by higher-resolution genotyping using 25-loci variable-number tandem repeat analysis (MLVA-25). Canonical SNP typing indicated that Turkish strains belonged to both the A.Br.003 linage and the Australian 94 lineage. In light of a recent analysis that placed the majority of Georgian *B. anthracis* isolates in one phylogenetic group, we screened the Turkish strains using a previously developed Georgian SNP panel. Minimal diversity was observed among the Kars strains within the Georgian SNP lineage: all 30 of these strains grouped with A.Br.026, ten strains were derived from A.Br.028, and only two isolates belonged to A.Br.029. According to the results of MLVA-25 genotyping, all 30 Turkish strains belong to two clusters. Cluster A is more diverse than cluster B. Our results suggest that *B. anthracis* strains originating from Georgia and the northeastern part of Turkey are genetically interrelated, which could be explained by the geographic proximity of the countries.

Keywords: *B. anthracis*; Especially Dangerous pathogens; MLVA and SNP genotyping

Introduction

The etiologic agent of anthrax, *Bacillus anthracis*, is a monomorphic member of a highly diverse group of endospore-forming bacteria. There are at least 51 known Bacillus species and many more of uncertain taxonomic status [1]. *B. anthracis* spores are typically found in soil and may be spread through contaminated dust, water, and plant and animal materials. The toxins produced by vegetative *B. anthracis* dictate its virulence and differ from the toxins produced by other Bacillus species.

Although anthrax is primarily a disease of herbivores, humans may contract anthrax directly or indirectly from animals [2]. The most common form of human anthrax, cutaneous anthrax, accounts for 95 to 99% of human cases worldwide and usually results from handling contaminated animal products. Infection occurs through a break in the skin and results in lesions on exposed regions of the body. After an incubation period of 2 to 3 days, a small papule appears, vesicles develop in a ring around the papule, and the papule subsequently ulcerates, dries, and blackens to form a distinctiveeschar. Less than 20% of untreated cases of cutaneous anthrax are fatal. In fatal cases, generalized symptoms may be mild (e.g., malaise and a slight fever) or absent before the sudden onset of acute illness, which is characterized by dyspnea, cyanosis, severe pyrexia, and disorientation followed by circulatory failure, shock, coma, and death in quick succession [3]. Concomitant with the severe signs of illness, the number of *B. anthracis* in the blood increases rapidly and reaches a maximum concentration during the last few hours of life.

Two other forms of human anthrax have been described. Gastrointestinal anthrax is caused by the consumption of contaminated animal products, and pulmonary anthrax occurs when *B. anthracis* spores are inhaled. Although rare, these forms of anthrax are much more severe than cutaneous anthrax because they are more likely to result in the rapid dissemination of bacteria to regional lymph nodes and the development of fatal septicemia.

Anthrax vaccines are available for animals and humans, but the disease remains endemic in many countries, particularly those without effective vaccination policies. *Bacillus anthracis* is extremely difficult to eradicate from endemic areas because its spores remain viable in soil for many years, and because bacterial persistence is not dependent on animal reservoirs [4].

In Georgia, anthrax is classified as endemic and has persisted for centuries [5]. During 2000 – 2012, there were 592 reported cases of human cutaneous anthrax in Georgia. 299 cases (51%) were classified as rural, 103 (17%) were peri-urban and 190 (32%) were urban [6]. Recent evidence suggests an increase in the incidence rate of infection in Georgia and in neighboring countries including thehyperendemic regions in Turkey [7].

Although rare in large parts of the world, *B. anthracis* infection presents a significant medical problem in the Kars region of Turkey, where human infection occurs amongst local farmers who live near their animals. From 1995 to 2005 there were 2,415 human cases of anthrax in Turkey of which 19.7% occurred in the area around Kars [8].

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DNA #	Strain ID	Year of strain isolation	Region	Source of speciment	[a]Van Ert et al 2007 canSNP group	[*]Group defined by canSNP assays published in Birdsell et al. 2012	GeoSNP group identified in this study published in Khmaladze et al. 2014
1	8347-G	2007	Tetri Tskaro	wash-out	A.Br.Aus94	A.Br.013/015	A.Br.027/028
2	8295-G	2007	Terjola	soil	A.Br.Aus94	A.Br.013/015	A.Br.029/030
3	9102-G	2008	Gardabani	blood	A.Br.Aus94	A.Br.013/015	A.Br.032/033
4	9099-G	2008	Gardabani	soil	A.Br.Aus94	A.Br.013/015	A.Br.032/033
5	9104-G	2008	Gardabani	ulcer	A.Br.Aus94	A.Br.013/015	A.Br.032/033
6	9107-G	2008	Gardabani	ulcer	A.Br.Aus94	A.Br.013/015	A.Br.028/029
7	9105-G	2008	Khobi	soil	A.Br.Aus94	A.Br.013/015	A.Br.028/029
8	89-G	2009	Tsalka	ulcer	A.Br.Aus94	A.Br.013/015	A.Br.028/029
9	52-G	2009	Tbilisi	meat	A.Br.Aus94	A.Br.013/015	A.Br.033
10	50-G	2009	Marneuli	bowel	A.Br.Aus94	A.Br.013/015	A.Br.032/033
11	91-G	2009	Tsalka	soil	A.Br.Aus94	A.Br.013/015	A.Br.028/029
12	154-G	2009	Rustavi	ulcer	A.Br.Aus94	A.Br.013/015	A.Br.028/029
13	1242-G	2002	Zugdidi	soil	A.Br.Aus94	A.Br.013/015	A.Br.029/030
14	1998-G	2002	Rustavi	ulcer	A.Br.Aus94	A.Br.013/015	A.Br.028/029
15	411-G	2001	Kutaisi	ulcer	A.Br.Aus94	A.Br.013/015	A.Br.027/028
16	406-G	2001	Zugdidi	soil	A.Br.Aus94	A.Br.013/015	A.Br.028/029
17	392-G	2001	Rustavi	Ulcer	A.Br.Aus94	A.Br.013/015	A.Br.027/028
18	368-G	2001	Kutaisi	ulcer	A.Br.Aus94	A.Br.013/015	A.Br.027/028
19	8670-G	1992	Sukhumi	soil	A.Br.Aus94	A.Br.013/015	A.Br.027/028
20	9630-G	2000	Akhalkalaki	ulcer	A.Br.Aus94	A.Br.013/015	A.Br.027/028
21	9450-G	1999	Zestaphoni	ulcer	A.Br.Aus94	A.Br.013/015	A.Br.028/029
22	8903-G	1997	Gardabani	soil	A.Br.Aus94	A.Br.013/015	A.Br.028/029
23	8889-G	1996	Gardabani	ulcer	A.Br.Aus94	A.Br.013/015	A.Br.028/029
24	8500-G	1991	Sukhumi	wash-out	A.Br.Aus94	A.Br.013/015	A.Br.027/028
25	8276-G	2007	Gardabani	soil	A.Br.Aus94	A.Br.013/015	A.Br.029/030
26	8263-G	2007	Kaspi	soil	A.Br.Aus94	A.Br.013/015	A.Br.028/029
27	7763-G	2007	Zestaponi	ulcer	A.Br.Aus94	A.Br.013/015	A.Br.028/029
28	7762-G	2007	Zestaphoni	ulcer	A.Br.Aus94	A.Br.013/015	A.Br.028/029
29	6671-G	2006	Gardabani	sheep intestine	A.Br.Aus94	A.Br.013/015	A.Br.029/030
30	6150-G	2006	Rustavi	ulcer	A.Br.Aus94	A.Br.013/015	A.Br.029/030

Table 1: Lineages of Georgian isolates used in the study. SNPs are defined by their positions in the B. *anthracis* genome.

There have been relatively few studies to characterize the strains of *B.anthracis* circulating Turkey [9,10], but an in-depth understanding of Turkish *B. anthracis* population is necessary to effectively identify strains and trace them to their origin. In addition, a more complete understanding of antigenic differences among Turkish strains could contribute to improved vaccine intervention strategies to curtail natural or weaponized *B. anthracis* outbreaks.

Studies carried out in Turkey and Georgia have sought to clarify genetic relationships among *B. anthracis* strains circulating in the region. In 2006, Merabishvili et al. used eight-loci variable-number tandem repeat analysis (MLVA-8) to determine the subtypes of 18 Georgian field-isolated and five *B. anthracis* vaccine strains (former Soviet Union (FSU) vaccines administered to livestock throughout the FSU). They found that these strains fell within the A.3.a subgroup (previously defined by Keim et al.) in two genotype clades shared with regional Turkish isolates [5,11]. Similarly, Durmaz et al. studied 251 B.anthracis strains isolated from human, animal, and environmental samples collected throughout Turkey and found a total of 12 distinct MLVA-25 A.3.a subtypes [11]. Ortatatli et al. examined the genetic diversity of 55 *B. anthracis* isolates from 16 distinct regions of Turkey [12] and identified three geographically related subgroups circulating in three distinct regions; genotype dispersal patterns were indicative of trans-boundary contamination from livestock. Khmaladze et al. screened multiple Georgian strains using 26 canonical single nucleotide polymorphism (can SNPs) assays, which placed these strains into eight newly identified groups within the A.Br.013/015 lineage [15]. Canonical SNP analysis is a phylogenetic approach used to identify SNPs that efficiently partition bacterial strains in genetic groups consistent with their recognized population structure.

Here we describe the use of can SNP analysis and MLVA to determine the subtypes of *B. anthracis* strains from Georgia and northeastern Turkey. Comparative analysis was conducted to get insight into the regional phylogenetic placement of the Georgian and Turkish strains, provide new insight on the evolutionary history, regional settlement and differentiation of *B. anthracis* strains of Caucasus region.

Materials and Methods

Bacillus anthracis strain culture and inactivation

In total, 60 *B. anthracis* samples were studied: 30 samples were provided by Kafkas University in Kars, Turkey and 30 samples were provided by the National Center for Disease Control and Public Health in Tbilisi, Georgia. *Bacillus anthracis* isolates from pure cultures grown on 5% Sheep Blood Agar (SBA) plates (Eliava Media Production, Georgia) were incubated at 37˚C for 24hours. Several loops of culture were transferred to 1.5-mL micro centrifuge tubes and heat-inactivated in an autoclave at 121˚C for 20 minutes [13].

DNA isolation and sterility testing

Sterile genomic DNA was extracted using QIAamp DNA Mini Kits (Qiagen, USA) according to the manufacturer's instructions. Purified DNA was divided into 100µL aliquots and stored at -20˚C pending analysis. DNA concentrations were measured using a NanoDrop 2000 (Thermo Fisher Scientific, USA).

We determined sample sterility by pipetting 5% of the final volume of the DNA and incubating at 37˚C in the same growth media used in bacterial culturing. To confirm sterility, at day +3 and day +7, 5µL of isolated DNA was placed on 5% SBA and incubated at 37˚C. If no growth was observed after 72hours at either time point, then the preparation was considered sterile. Primary and secondary containers were decontaminated with 1% sodium hypochlorite for 30

NDA #	strains ID	Year of strain Isolation	Region	Source of speciment	[9]Van Ert et al 2007 canSNP group	[8]Group defined by canSNP assays published in Birdsell et al. 2012	GeoSNP group identified in this study published in Khmaladze et al. 2014
1	K-2	<2004	Kars	Cattle	A.Br.Aus94	A.Br.013/015	A.Br.028/029
2	K-28	<2004	Kars	Cattle	A.Br.Aus94	A.Br.013/015	A.Br.026/027
3	K-44	2006	Kars	Cattle	A.Br.Aus94	A.Br.013/015	A.Br.026/027
4	K-51	2010	Kars	Cattle	A.Br.Aus94	A.Br.013/015	A.Br.029/030
5	K-52	2005	Kars	Cattle	A.Br.Aus94	A.Br.013/015	A.Br.028/029
6	K-60	2007	Kars	Cattle	A.Br.Aus94	A.Br.013/015	A.Br.026/027
7	K-62	2008	Kars	Cattle	A.Br.Aus94	A.Br.013/015	A.Br.026/027
8	K-68	2012	Kars	Cattle	A.Br.Aus94	A.Br.013/015	A.Br.028/029
9	K-78	2013	Kars	Cattle	A.Br.Aus94	A.Br.013/015	A.Br.028/029
10	K-80	2014	Kars	Cattle	A.Br.Aus94	A.Br.013/015	A.Br.026/027
11	K-86	2004	Kars	Cattle	A.Br.Aus94	A.Br.013/015	A.Br.026/027
12	K-98	2009	Kars	Cattle	A.Br.Aus94	A.Br.013/015	A.Br.028/029
13	K-100	2011	Kars	Cattle	A.Br.Aus94	A.Br.013/015	A.Br.026/027
14	K-107	2004	Kars	Sheep	A.Br.Aus94	A.Br.013/015	A.Br.026/027
15	K-116	<2004	Kars	Sheep	A.Br.Aus94	A.Br.013/015	A.Br.026/027
16	K-132	2012	Kars	Sheep	A.Br.Aus94	A.Br.013/015	A.Br.028/029
17	K-139	2006	Kars	Sheep	A.Br.Aus94	A.Br.013/015	A.Br.026/027
18	K-145	2008	Kars	Sheep	A.Br.Aus94	A.Br.013/015	A.Br.026/027
19	K-146	2013	Kars	Human	A.Br.Aus94	A.Br.013/015	A.Br.026/027
20	K-149	2013	Kars	Dog	A.Br.Aus94	A.Br.013/015	A.Br.028/029
21	K-150	2012	Kars	Environmental	A.Br.Aus94	A.Br.013/015	A.Br.026/027
22	K-156	2012	Kars	Environmental	A.Br.Aus94	A.Br.013/015	A.Br.029/030
23	K-160	2013	Kars	Environmental	A.Br.Aus94	A.Br.013/015	A.Br.026/027
24	K-173	2013	Kars	Environmental	A.Br.Aus94	A.Br.013/015	A.Br.026/027
25	K-183	2014	Kars	Environmental	A.Br.Aus94	A.Br.013/015	A.Br.026/027
26	K-199	2012	Kars	Environmental	A.Br.Aus94	A.Br.013/015	A.Br.028/029
27	K-204	2013	Kars	Environmental	A.Br.Aus94	A.Br.013/015	A.Br.026/027
28	K-211	2014	Kars	Environmental	A.Br.Aus94	A.Br.013/015	A.Br.028/029
29	K-215	2009	Kars	Environmental	A.Br.Aus94	A.Br.013/015	A.Br.028/029
30	K-220	2012	Kars	Environmental	A.Br.Aus94	A.Br.013/015	A.Br.026/027

Table 2: Lineages of Turkish isolates used in the study. SNPs are defined by their positions in the *B. anthracis* genome.

minutes, and stored at -20°C. After surface decontamination, sterile samples could be handled under biosafety level-1 containment.

SNP analysis

To conduct can SNP analysis of *B. anthracis* strains, a specific SNP melt analysis of mismatch amplification mutation assay (melt-MAMA) was used [14]. Primer concentrations were adjusted to 100pmol/µL in Tris-EDTA buffer. Each primer was diluted to 10pmol/µL with distilled water to create a working stock.

Synthetic, allele-specific, positive control templates were created by conventional PCR. Primer mixes contained 10pmol/µL of ancestral allele primer (SA), derived-allele primer (SD) and reverse primer (SC). Each 40-µL, single-primer-set PCR reaction contained 1µL of primer mix; 36µL Platinum PCR SuperMix and 2.0µL genomic DNA (> 5ng/µL). Conventional PCR products were verified by electrophoresis on 2% agarose gel in 1X Tris-acetate-EDTA buffer at 10V/cm for two hours. Original PCR products amplified using SD-SC and SA-SC primers were diluted 10,000X for use as synthetic allele-specific positive control templates for determination of melting temperature (T_m) for both SD and SA with SC. Real-Time PCR amplification followed with melt analysis was then performed using genomic DNA and the primer mixes on CFX 96 Real-Time PCR detection system (Bio-Rad). Each 10-µL PCR reaction contained of 1µL primer mix for SD, SA, and SC (10pmol/µL); 5µL of 2 X SYBR Green master mixes; 1µL of genomic DNA (10ng/µL); and 3µLddH_2O. The Tm from each sample was compared to the appropriate T_m reference table to determine the SNP base call.

MLVA-25 analysis

Forward and reverse primers were combined and diluted to 5µM or 10µM to create working primer stocks. The DNA was amplified in four multiplex PCR reactions (multiplex A, B, C and D). The PCR master mix was prepared with 7.55µL molecular-grade water (ddH_2O), 1.5µL 10X PCR buffer, 2.25µL primers in total, 1.5µL 50mM$MgCl_2$, 1µL dNTPs (2.5mM each), and 0.2µL Platinum Taq DNA Polymerase.

Each PCR contained 14µL of master mix and 1µL of sample DNA; 1µL of ddH_2O served as the negative control. After heat-denaturing the DNA for 5minutes at 95°C, PCR reactions were performed with the following cycling conditions: 38 cycles of 30 sec at 95°C, 30 sec at 55°C and 1 min at 72°C. 7min at 72°C, final extension 5min at 72°C and 4°C hold.

After amplification, 2µL of each PCR reaction was diluted 100-fold in 198µL of ddH_2O. A denaturation solution/sizing standard solution was prepared from 18.7µL of HiDi Formamide and 0.3µL of 1200LIZ size standard; 19µL of the resulting solution and 1µL of the diluted multiplex samples were added to the wells of an ABI platform-compatible plate, e.g., MicroAmp Optical 96-well Reaction Plate (life technologies). Samples were denatured in a GeneAmp PCR System 9700 (Applied Biosystems) for five minutes at 95°C and then placed on ice for three to five minutes. Reactions were run on an ABI 3130xl instrument (Thermo Fisher Scientific) and fragment analysis was performed with GeneScan and GeneMapper software packages (Applied Biosystems). GeneMapper software was used to analyze electropherograms and score VNTR sizes.

Results

Canonical SNP typing of *B. anthracis* strains from Turkey and Georgia revealed that Turkish strains belonged to *B. anthracis* group A.Br.003 and the Australian 94 lineages. The lineages of the Georgian isolates used in the study are shown Table 1. The lineages of Turkish

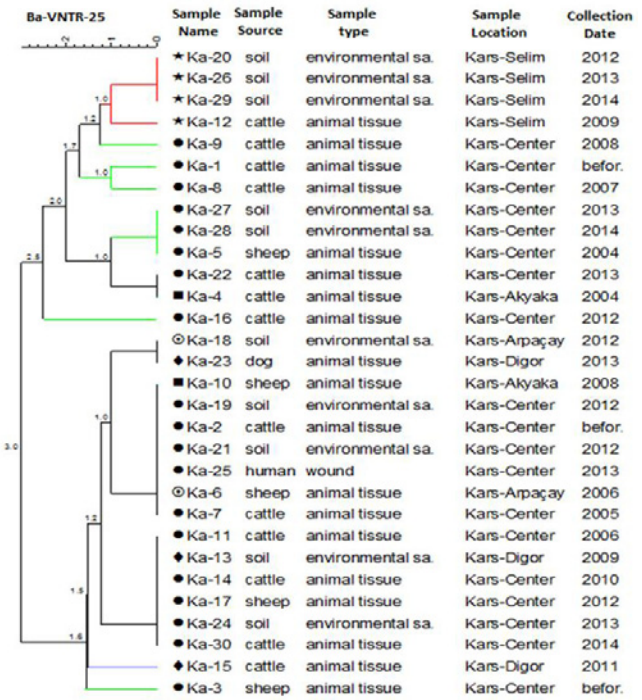

Figure 1: Dendrogram depicting results of MLVA-25 analysis of *B. anthracis* specimens from Kars region of Turkey.

isolates used in the study are shown in Table 2. The SNPs are defined by their positions in the *B. anthracis* genome as shown in these two tables. Given the results of our recent study, which indicated that the majority of Georgian *B. anthracis* isolates belong to the same phylogenetic group, the Turkish strains were screened against the Georgian SNP panels as described by Khmaladze et al. [15]. Some diversity was observed among the Turkish strains within the Georgian SNP lineage: all 30 of these strains grouped with A.Br.026, ten strains were derived from A.Br.028, and only two isolates belonged to A.Br.029. Figure 1 is a dendrogram depicting the results of MLVA-25 analysis of *B. anthracis* specimens from Kars region of Turkey. Figure 2 is a comparison of MLVA-25 data for *B. anthracis* specimens derived from Georgia and Kars region of Turkey. According to the results of MLVA-25 genotyping, all 30 Turkish strains belong to two clusters. Cluster A is more diverse than cluster B, which could be explained by the sample size and location.

Discussion

In this study, the MLVA-25 data from the thirty Turkish isolates and thirty Georgian isolates and the canonical SNP typing indicate that Turkish strains belonged to both the A.Br.003 linage and the Australian 94 lineages. Even though minimal diversity was observed among the Kars strains within the Georgian SNP lineage: all 30 of these strains grouped with A.Br.026, ten strains were derived from A.Br.028, and only two isolates belonged to A.Br.029. According to the results of MLVA-25 genotyping, all 30 Turkish strains belong to two clusters. Cluster A is more diverse than cluster B. Our results suggest that *B. anthracis* strains from Georgia and the northeastern part of Turkey are genetically interrelated.

The global genetic population structure of *B. anthracis* suggests

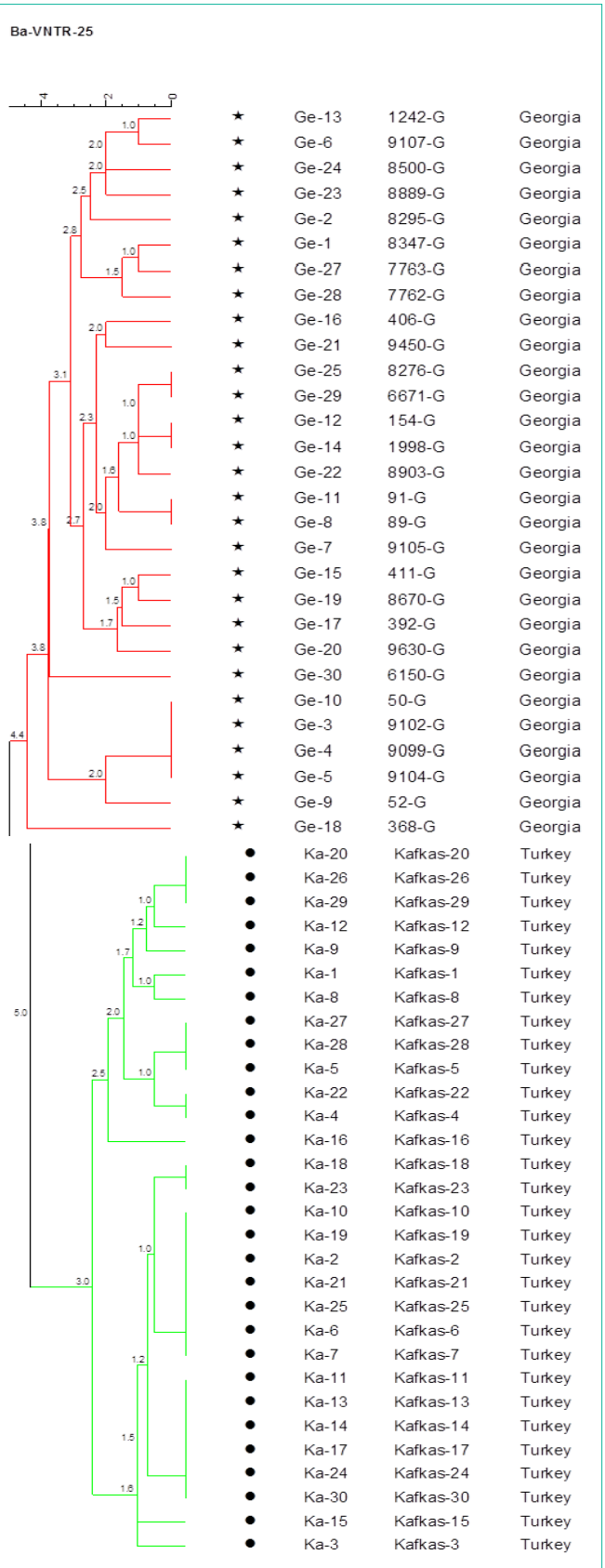

Figure 2: Comparison of MLVA-25 data for *B. anthracis* specimens derived from Georgia and Kars region of Turkey.

that human activities have played a key role in the proliferation and dispersal of the bacteria. The estimated divergence of the A lineage of *B. anthracis* occurred during a period of human history that was marked by major agricultural developments. As domestication and mammal husbandry of large mammals expended beyond centers in Eurasia and North Africa, animals were subsequently transported along major trade routes such as the Silk Road running through Georgia and eastern Turkey. *B. anthracis* is considered to have a high degree of genetic homogeneity, which makes it difficult to discriminate among specimens. Genetic homogeneity is driven by the high spore survival capacity developed by *B. anthracis* during its evolution. The genetic homogeneity of Georgian and Turkish *B. anthracis* strains is likely the result of migration of the pathogen across the Georgia-Turkey border over time.

More recently the incidence of human anthrax has increased in Georgia but not in the neighboring Kars region of Turkey. The fact that closely related strains of the same lineage are prevalent in both regions indicates that these differences in human disease trends reflect differences in agricultural and social practices rather than in the inherent virulence of the pathogen. Indeed, a recent study from Azerbaijan found that the introduction of an effective prophylactic animal vaccination program markedly reduced the incidence of the disease in both animals and humans [16].

Conflict of Interest

This research described herein was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

E.Kh. experimentation, protocol optimization, W.S. Experimentation, protocol optimization, E.Z. experimentation, protocol optimization, F.B. experimentation, sample preparation, M.S. experimentation, sample preparation M.N. experimentation, protocol optimization, data analysis, L.B. data analysis R.O. writing the manuscript, technical editing A.K. experimentation, project manager, manuscript writing.

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